Epithelial microRNA-9a Regulates Dendrite Growth Through Fmi-Gq Signaling in *Drosophila* Sensory Neurons

Yan Wang,[1,2]* Huan Wang,[1,3]* Xiaoting Li,[1,3] Yan Li[1]

[1] State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

[2] Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing 100101, China

[3] University of Chinese Academy of Sciences, Beijing 100049, China

Received 17 January 2015; revised 15 April 2015; accepted 24 May 2015

ABSTRACT: **microRNA-9 (*miR-9*) is highly expressed in the nervous system across species and plays essential roles in neurogenesis and axon growth; however, little is known about the mechanisms that link *miR-9* with dendrite growth. Using an *in vivo* model of *Drosophila* class I dendrite arborization (da) neurons, we show that *miR-9a*, a *Drosophila* homolog of mammalian *miR-9*, downregulates the cadherin protein Flamingo (Fmi) thereby attenuating dendrite development in a non-cell autonomous manner. In *miR-9a* knockout mutants, the dendrite length of a sensory neuron ddaE was significantly increased. Intriguingly, *miR-9a* is specifically expressed in epithelial cells but not in neurons, thus the expression of epithelial but not neuronal Fmi is greatly elevated in *miR-9a* mutants. In contrast, overexpression of Fmi in the neuron resulted in a reduction in dendrite growth, suggesting that neuronal Fmi plays a suppressive role in dendrite growth, and that increased epithelial Fmi might promote dendrite growth by competitively binding to neuronal Fmi. Fmi has been proposed as a G protein-coupled receptor (GPCR), we find that neuronal G protein Gαq (Gq), but not Go, may function downstream of Fmi to negatively regulate dendrite growth. Taken together, our results reveal a novel function of *miR-9a* in dendrite morphogenesis. Moreover, we suggest that Gq might mediate the intercellular signal of Fmi in neurons to suppress dendrite growth. Our findings provide novel insights into the complex regulatory mechanisms of microRNAs in dendrite development, and further reveal the interplay between the different components of Fmi, functioning in cadherin adhesion and GPCR signalling.** Develop Neurobiol 76: 225–237, 2016

Keywords: **microRNA-9a; non-cell autonomous; Flamingo; Gq; dendrite growth**

INTRODUCTION

Dendrites are vital structures in neurons for information sampling and processing. Numerous molecules, including cell surface receptors, signaling cascades, transcription factors, and cytoskeletal components, are known to regulate dendrite growth and patterning (Jan and Jan, 2010); however, a possible involvement of microRNAs (miRNAs) in dendrite morphogenesis remains to be elucidated. miRNAs are noncoding RNAs with approximately 21–23 nucleotides, which

Correspondence to: Y. Li; (liyan@ibp.ac.cn) and Y. Wang; (wangy@mail.biols.ac.cn).

*Y.W. and H.W. contributed equally to this work.

Contract grant sponsor: National Science Foundation of China; contract grant number: 31070956, 91132709, 31130027, and 81000559.

Contract grant sponsor: One Hundred Talents Project of the CAS; contract grant number: KSCX2-YW-R-156.

Additional Supporting Information may be found in the online version of this article.

Published online 8 June 2015 in Wiley Online Library (wileyonlinelibrary.com).

DOI 10.1002/dneu.22309

posttranscriptionally repress target mRNAs by binding to their 3' untranslated regions (3' UTR) (Ambros, 2001; Bartel, 2009). miRNAs play important roles in axonal devleopment (Vo et al., 2010; Iyengar et al., 2014), as well as spine formation and plasticity (Schratt et al., 2006; Wayman et al., 2008; Siegel et al., 2009; Lee et al., 2012; Jian et al., 2014); however, there are few studies on miRNAs function in dendrite growth. One study in cultured cortical and hippocampal neurons revealed that *miR-132* is required for activity-dependent dendritic growth (Magill et al., 2010). One *in vivo* study on *Drosophila* sensory neurons reported that miRNA *bantam* (*ban*), which is expressed in epithelial cells, is involved in the regulation of dendrite scaling (Parrish et al., 2009). Despite their importance in posttranscriptional regulation, the precise roles of miRNAs in dendrite development, together with the underlying mechanisms, have not been explored in great detail.

Drosophila microRNA-9 (*miR-9a*) belongs to the *miR-9* family of miRNAs, which is implicated in controlling the fundamental biological processes during neural development (Yuva-Aydemir et al., 2011). *miR-9* was shown recently to define the neurogenic boundaries in *Drosophila* and zebrafish and to regulate the proliferation, migration and differentiation of neural progenitor cells (NPCs) in vertebrates (Yuva-Aydemir et al., 2011). In *Drosophila*, *miR-9a* controls the accurate specification of NPCs by downregulating the transcription factor Senseless (Sens) in epithelial cells (Li et al., 2006). In the embryonic brain of zebrafish, *miR-9* prevents cells in the brain region adjacent to midbrain-hindbrain boundary (MHB) from adopting the MHB fate through suppressing transcription factor her5 (Coolen et al., 2012). During brain development of Xenopus, *miR-9* limits NPC proliferation through a transcriptional regulator of Hes family, namely hairy 1 (Bonev et al., 2011). In postmitotic neurons, *miR-9* has also been found to inhibit axon extension and branching by targeting Map1b, an important protein for microtubule stability in a mouse model (Dajas-Bailador et al., 2012). However, the function of *miR-9* in dendrite development has so far eluded identification.

Drosophila Flamingo (Fmi, also known as *stan*) has three orthologs in mammals, named Cadherin EGF LAG Seven-pass G-type Receptor 1–3 (Celsr1-3), all belonging to an evolutionally conserved cadherin superfamily, and possessing large ectodomains in N-terminal and transmembrane domain in C-terminal (Usui et al., 1999). In the nervous system of both flies and mammals, Fmi has been found to regulate neurite morphogenesis (Gao et al., 2000; Shima et al., 2007). As cadherin domains mediate cell–cell adhesion, Fmi is thought to be involved in axon navigation through homophilic adhesion between pioneer and follower axons in *C. elegans* (Steimel et al., 2010). Similar to other seven-pass transmembrane domain proteins, Fmi was also proposed to function as a GPCR (Shima et al., 2007; Berger-Muller and Suzuki, 2011). However, the G protein coupled to Fmi/Celsr1-3 has not been identified to date.

In this study, we set out to investigate the precise function of *miR-9a* in dendrite development, for which we used an *in vivo* model consisting of a *Drosophila* class I dendrite arborization (da) neuron, ddaE, together with its neighboring epithelial cells. Our data suggest that *miR-9a* is expressed in epithelial cells and not, however, in neurons, and therefore inhibits dendrite development in a non-cell autonomous manner. Moreover, we found that Fmi was downregulated by *miR-9a*. We thus address whether the function of *miR-9a* in dendrite development is mediated by Fmi, and which function of Fmi, homophilic adhesion or signal transduction, is required for dendrite growth.

MATERIALS AND METHODS

Fly Stocks

All flies were maintained at 25°C. Fly strains of $Gal4^{2\text{-}21}$, $miR\text{-}9a^{E39}$, $miR\text{-}9a^{J22}$, and UAS-*miR-9a* were kindly provided by Dr. Fen-Biao Gao (University of Massachusetts Medical School). *miR-9a* sensor was obtained from Eric C. Lai (Memorial Sloan Kettering Cancer Center). UAS- Fmi, UAS-FmiΔN::EYFP and UAS-FmiΔC::EYFP were from Dr. Tadashi Uemura (Kyoto University). UAS-Go^{GTP} was from Dr. Andrew Tomlinson (Columbia University). $Gal4^{e22c}$ was from Dr. Michael J Galko (University of Texas MD Anderson Cancer Center). The following fly strains were purchased from the Bloomington stock center: *fmi* mutant $stan^{f00907}$, UAS-Gq, UAS-Gq^{AC}, UAS-Gq^{RNAi}, UAS-fmi^{RNAi}. The following RNAi fly lines were from Vienna *Drosophila* RNAi Center: UAS-fz^{RNAi}, UAS-dsh^{RNAi}, and UAS-Go^{RNAi}. UAS-$vang^{RNAi}$ was kindly provided by Dr. Jian-Quan Ni (Tsinghua Fly Center, School of Medicine, Tsinghua University).

Immunohistochemistry

Immunohistochemistry was performed in embryos and on the body walls of third instar larvae according to standard protocols (Wu and Luo, 2006). Briefly, Embryos were collected and aged to the stages specified in the text. After dechorionation in 50% bleach, embryos were fixed in equal volumes of heptane and 4% formaldehyde in Phosphate Buffered Saline (PBS) at room temperatures (RT) for 40 min. The devitellinized embryos were rinsed in methanol

and then washed in 50% methanol/50% PBST and then in 0.3% PBST. Fixed embryos were rinsed and blocked with 5% normal goat serum for 1 h. Body walls of third instar larvae were gently dissected without being stretched or pressured, and then fixed with 4% paraformaldehyde (Electron Microscopy Sciences) at RT for 40 min. Samples were incubated in primary and then secondary antibodies at 4°C overnight. Mouse monoclonal antibodies were from Developmental Studies Hybridoma Bank (DSHB, University of Iowa): Fmi (1:200), Fasciclin III (fasIII, 1:500), 22C10 (1:500). Secondary antibody was Alexa Fluor® 555 Donkey anti-mouse IgG (Invitrogen, 1:1000).

Confocal Microscopy

After immunostaining, samples were mounted with VECTASHIELD® mounting media (Vector Laboratories). Third instar larvae with class I sensory neurons labeled by $Gal4^{2\text{-}21}$, UAS-mGFP were rinsed in PBS, and then immobilized using a cover slip. All embryos were rotated to the anterior-left and dorsal-up. Confocal images were obtained with a Leica SPE or a SP5 II MP microscope. The surface and lower layers were scanned for epithelial and neuronal signals of Fmi and *miR-9a* sensor, respectively. Dendrite morphology was analyzed using NIS-Element D 3.0 software, and fluorescent intensities were measured using Image J (National Institutes of Health).

Quantitative Real Time PCR (qRT-PCR)

qRT-PCR was performed to determine *fmi* mRNA expression levels. Briefly, total RNA was extracted from 5 third instar larvae with TRIzol® (Invitrogen). Reverse transcription was performed using 2 μg RNA per sample with PrimeScript RT Master Mix (TaKaRa, Japan). qRT-PCR validation was performed using the Maxima® SYBR® Green qPCR Master Mix kit (CWBIO, China) according to the manufacturer's instructions, in ABI Prism 7500 Sequence Detection System (Applied Biosystems). The *rp49* gene was used as an internal control (forward primer, 3'-CCAAGGACTTCATCCGCCACC-5'; reverse primer, 3'-GCGGGTGCGCTTGTT CGATCC-5'). For *fmi*, the forward primer was 3'-AATCCGGGCTTGATGGGAAG-5', and the reverse primer was 3'-TCCCAGGACCAGTCGGTATC-5'. Quantification of *fmi* expression was performed using the delta Ct method.

Statistical Analysis

The parameters for estimating the dendrite morphology of ddaE neuron were defined as following: (1) total length-PDs, the total length of the primary dendrites; (2) length of D-V, the dendritic coverage range at D-V direction; and (3) length of A-P, the dendritic coverage range at A-P direction. For measurements of Fmi intensity, the fluorescent intensity in a $10.1 \times 31\ \mu m^2$ region covering all dorsal sensory neurons of a semisegment was quantified using Image J (National Institutes of Health).

Statistical analysis was performed using Student's *t*-test and one-way analysis of variance (ANOVA), followed by a *post hoc* Tukey's test as comparison. Quantification data are shown as mean values ± s.e.m; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and N.S. indicates that no significant changes were observed.

RESULTS

Epithelial *miR-9a* Regulates Dendrite Growth in a Non-Cell Autonomous Manner

In *Drosophila*, *miR-9a* has been reported to play an important role in the specification of neuronal precursors (Li et al., 2006). In *Drosophila* sensory neuron ddaE, dendrites first elongate at dorsal-ventral (D-V) direction as the primary dendrites (PDs), and those at anterior-posterior (A-P) direction are second-order branches, sprouting from the side of the first-order branches (Sugimura et al., 2003). To investigate the function of *miR-9a* in dendrite development in postmitotic neurons, we examined the dendrite morphology of ddaE neuron in *miR-9a* mutant at third instar larval stage. We calculated the length of dendrites in both D-V and A-P direction [Fig. 1(D)], and found that the coverage range of dendrites in D-V direction (length-DV) was significantly increased in two *miR-9a* mutants, $miR\text{-}9a^{E39}$, and $miR\text{-}9a^{J22}$, when compared to wild type flies. However, no changes were observed in the A-P direction [length-AP, Fig. 1(A–F)]. We then specifically measured the total length of two PDs [total length-PDs, Fig. 1(D)]; and consistently observed an increment in two *miR-9a* mutants [Fig. 1(G)]. The abnormal growth of primary dendrites in *miR-9a* mutants suggested that *miR-9a* plays an inhibitory role in dendrite growth.

We next examined *miR-9a* expression using a *miR-9a* sensor approach, which contained two antisense target sites of *miR-9a* together with *tub-GFP* (Bejarano et al., 2010). We detected a strong GFP signal in the dorsal regions of stage 13–14 embryos [Fig. 1(H)], and most of the signals merged very well with the dorsal cluster sensory neurons (stained with 22C10, red signal) [Fig. 1(H–J)], indicating the absence of *miR-9a* expression in these neurons. In contrast, little GFP signal was detected in the epithelium (stained with fas III, red signal) [Fig. 1(K–M)], indicating strong and extensive expression of *miR-9a* in epithelial cells. This expression pattern of *miR-9a* was in agreement with previous findings in early-stage embryos (Stark et al., 2005; Li et al., 2006). Together with the overgrowth phenotypes of the dendrites in the *miR-9a* mutants, our results suggested that *miR-9a* has a non-cell autonomous effect in regulating dendrite development.

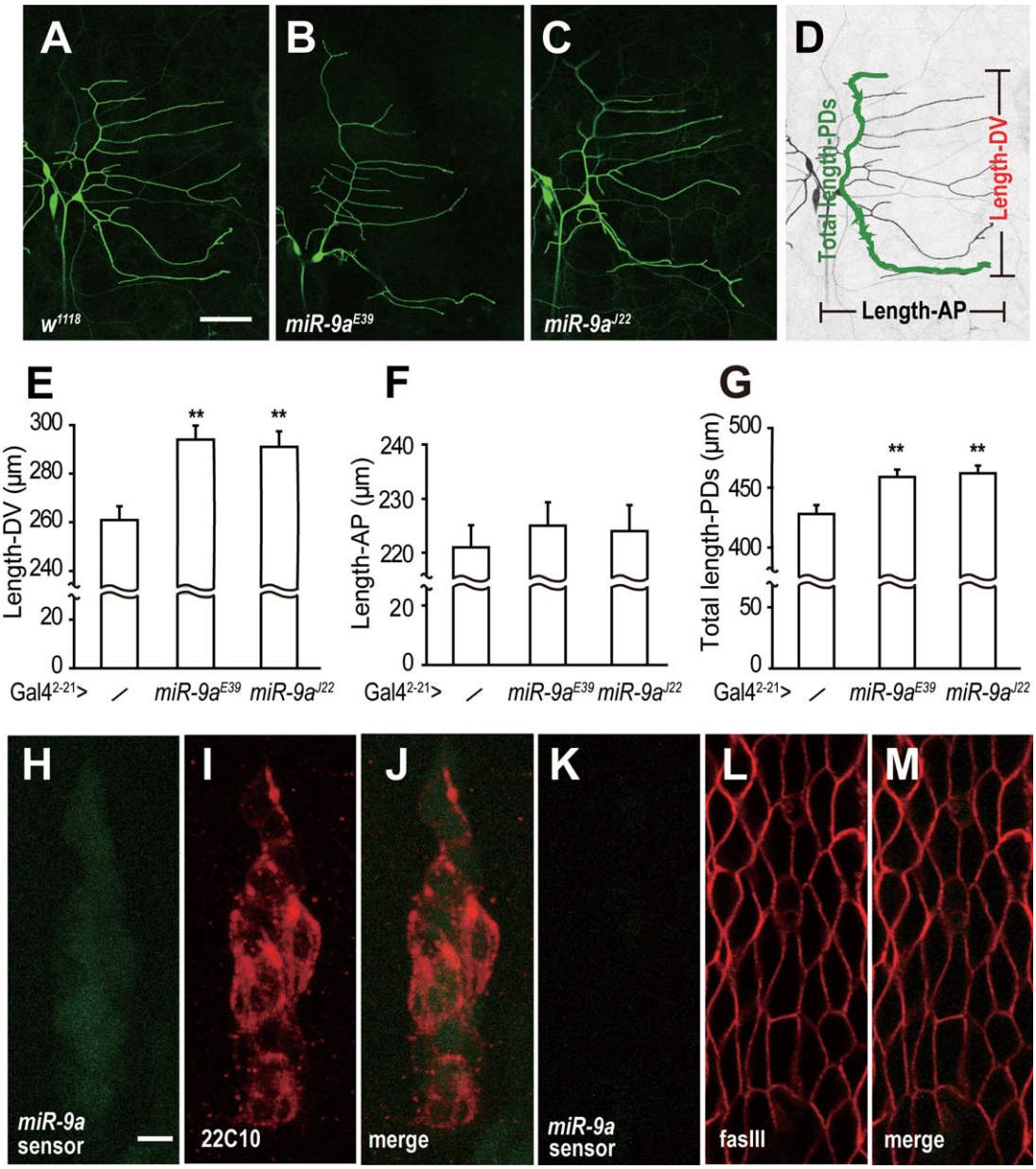

Figure 1 *miR-9a* non-cell autonomously regulates dendrite outgrowth. A–C: A representative image of ddaE neuron in wild type w^{1118}, *miR-9a*^{E39}, and *miR-9a*^{J22} mutant at third instar larva stage. D: The cartoon shows the dendrite morphology and the parameters quantified in corresponding panel. E–G: Quantitative analysis shows that both length-DV and total length-PDs of ddaE neurons in *miR-9a* mutants are significantly increased relative to wild type. The length-AP of the neurons remains unchanged. H–J: Expression of *miR-9a* is absent in neurons at embryonic stage 14. K–M: High expression of *miR-9a* in epithelial cells at embryonic stage 14. Scale bar, 50 μm in A, 5 μm in H. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

miR-9a Indirectly Regulates Dendrite Growth via Epithelial Flamingo

To investigate the downstream targets of *miR-9a* in dendrite growth, we performed sequence analysis and found a potential binding site for *miR-9a* in the 3'UTR of *fmi* (Supporting Information Fig. S1), suggesting that Fmi may be a downstream target of *miR-9a*. We thus examined the expression levels of Fmi in both epithelial cells and neurons of *miR-9a* mutants. Our analysis of *miR-9a*^{E39} mutants at embryonic stage 14 revealed a significant increase

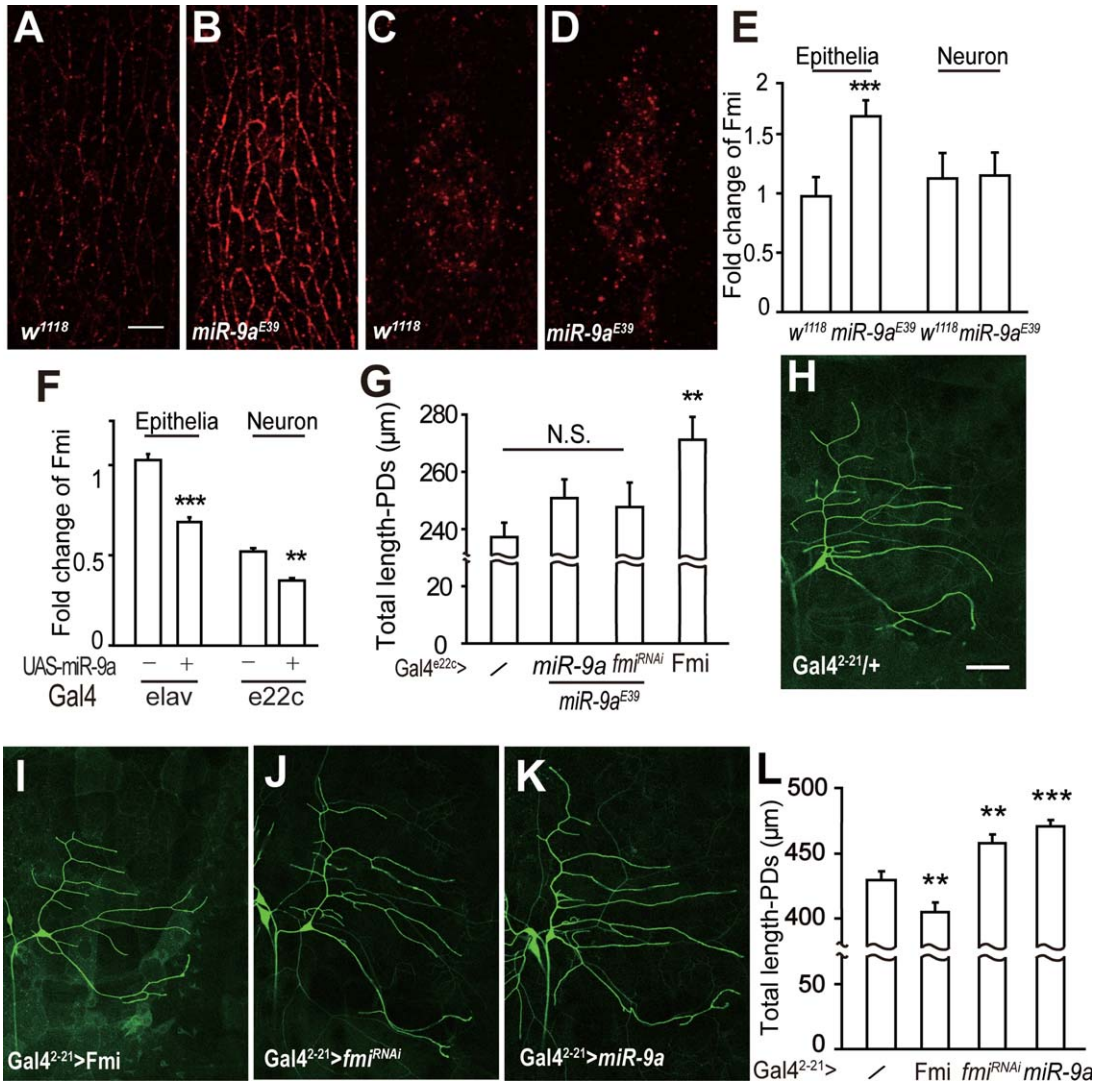

Figure 2 Fmi functions as a target of *miR-9a* in dendrite outgrowth. A–E: *miR-9a*^{E39} mutants display enhanced Fmi expression in epithelial cells (A, B, and E), but not in neurons (C–E). F: Quantitative analysis results show that overexpression of *miR-9a* in neurons (via elav-Gal4) and in epithelial cells (via Gal4^{e22c}) results in drastic reduction of Fmi expression. G: Both overexpression of *miR-9a* and downregulation of *fmi* in the epithelia restore the dendrite overgrowth phenotype in *miR-9a* mutants. Overexpression of Fmi in the epithelia results in an overgrowth phenotype. H–L: Overexpressing *miR-9a* or knockdown of *fmi* in the neurons by Gal4$^{2\text{-}21}$ results in increments of total length-PDs of ddaE neuron. Upregulation of Fmi in the neurons leads to a significant reduction in total length-PDs of ddaE neuron. Scale bar, 5 μm in A, 50 μm in I. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in Fmi expression, but intriguingly only in epithelial cells, but not in neurons [Fig. 2(A–E)]. To test whether Fmi was regulated by *miR-9a*, we ectopically overexpressed *miR-9a* in neurons (via elav-Gal4) and overexpressed *miR-9a* in epithelial cells (via Gal4^{e22c}). Remarkably, we detected a drastic reduction in Fmi expression in both cell types [Fig. 2(F) and Supporting Information Fig. S2(A–D)], suggesting the suppressive role of *miR-9a* in Fmi expression. To verify the role of epithelial *miR-9a* in dendrite growth, we overexpressed *miR-9a* in the epithelia of *miR-9a* mutants, and found that the dendrite overgrowth phenotype was rescued [Fig. 2(G) and Supporting Information Fig. S2(F)]. To see if epithelial Fmi was responsible for the dendrite phenotype in the neurons, we knocked down *fmi* in epithelial cells by RNA interference [Supporting Information Fig. S2(J,K)], which also rescued the

dendrite overgrowth phenotype observed in the *miR-9a* mutant [Fig. 2(G) and Supporting Information Fig. S2(G)]. Moreover, overexpressing Fmi in the epithelia [Supporting Information Fig. S2(I–K)] resembled the dendrite phenotype of ddaE neurons in *miR-9a* mutant [Fig. 2(G) and Supporting Information Fig. S2(H)]. Together, these findings suggested that Fmi is down-regulated by *miR-9a*, a process which may mediate *miR-9a* function in controlling dendrite growth.

Epithelial and Neuronal Fmi Perform Opposite Functions in Dendrite Growth

Flamingo/Celsr belongs to the atypical cadherin superfamily (Usui et al., 1999). Homophilic binding of Fmi through its cadherin domains at dendrodendritic interfaces triggers avoidance between dendritic terminals (Kimura et al., 2006). To test whether the overgrowth phenotype in *miR-9a* mutants was due to enhanced homophilic binding between epithelial and neuronal Fmi, we overexpressed Fmi in the neurons, and found a significant reduction in total length-PDs and length-DV [Fig. 2(H–L) and Supporting Information Fig. S3(A)]. Notably, enhanced Fmi expression in epithelial *miR-9a* mutants resulted in dendrite overgrowth, whereas upregulation of Fmi in the neurons led to decreased dendrite length. Moreover, knocking-down *fmi* or expressing *miR-9a* in ddaE neurons by $Gal4^{2\text{-}21}$ both resulted in dendrite overgrowth phenotype characterized by increased total length-PDs [Fig. 2(J–L)] and length-DV [Supporting Information Fig. S3(A)]. Thus, we speculated that neuronal Fmi negatively regulated dendrite growth, while excessive epithelial Fmi in *miR-9a* mutants competitively interfered with the function of neuronal Fmi through homophilic binding.

To investigate the posttranscriptional regulation of *miR-9a* in Fmi expression and thus its involvement in dendrite growth, we then used a homozygous viable *fmi* mutant $stan^{f00907}$ with an insertion just before its 3'UTR (Thibault et al., 2004), which displayed reduced Fmi expression in both neurons and epithelia [Fig. 3(A–G) and Supporting Information Fig. S2(L)]. We generated the double mutant of $stan^{f00907}$;$miR\text{-}9a^{E39}$, in which the levels of Fmi protein as examined by immunofluorescence and *fmi* mRNA as examined by qRT-PCR were similar to that of $stan^{f00907}$ [Fig. 3(A–G) and Supporting Information Fig. S2(L)]. Therefore, our results indicated that following the disruption of 3'UTR in this mutant, *fmi* expression is no longer suppressed by *miR-9a*.

In addition, we found in the $stan^{f00907}$ mutant, that primary dendrites of ddaE neurons were significantly increased [Fig. 3(J,M)], which could be fully rescued by neuron-specifically expressing Fmi in a $stan^{f00907}$ mutant background [Fig. 3(K,M)] and Supporting Information Fig. S3(B)]. Furthermore, in the $stan^{f00907}$; $miR\text{-}9a^{E39}$ mutant, dendrite growth was increased to similar levels observed in either of the two mutants [Fig. 3(H–M) and Supporting Information Fig. S3(B)]. Together, these results supported our hypothesis that neuronal Fmi plays a dominant role in dendrite growth, and that *miR-9a* regulates dendrite growth through controlling epithelial Fmi expression, thereby allowing the proper functioning of neuronal Fmi.

PCP Pathway Is not Required for Fmi-Mediated Dendrite Growth

Fmi is a core molecular in the planar cell polarity (PCP) pathway (Usui et al., 1999; Shimada et al., 2001; Strutt, 2002). To investigate whether PCP pathway is involved in Fmi-mediated dendrite growth, we knocked down *frizzled (fz)* in class I dorsal sensory neurons by RNA interference (RNAi). Our results showed that lack of *fz* had no effect on the total length-PDs or length-DV of ddaE neurons [Fig. 4(A–E)]. Similarly, both length-DV and total length-PDs of ddaE neuron in $Dishevelled^{RNAi}$ (Dsh^{RNAi}) and $Van\ Gogh^{RNAi}$ ($Vang^{RNAi}$) larvae remained normal [Fig. 4(A–E) and Supporting Information Fig. S3(C)], suggesting that Dsh and Vang did not affect dendrite growth. In addition, we found that neither overexpression nor knockdown Gαo (Go), a known Fz-coupled G protein, had any effect on dendrite growth [Fig. 4(F–H) and Supporting Information Fig. S3(D)]. Together, these results suggest that the PCP pathway is not required for Fmi-mediated dendrite growth.

Both Cadherin Repeats and Cytoplasmic Tail Are Required for Fmi-Mediated Dendrite Growth

Both N-terminal cadherin repeats (N-cadherin) and cytoplasmic tail (C-tail) of Fmi have been suggested earlier to play an important role in dendrite patterning by structure and functional analyses (Kimura et al., 2006; Shima et al., 2007). As FmiΔC lacks the intracellular C-tail of Fmi; whereas FmiΔN lacks conserved extracellular motifs of Fmi, we thus used them to explore the functions of N-cadherin and C-tail during dendrite growth (Kimura et al., 2006). Overexpression of Fmi in the neurons

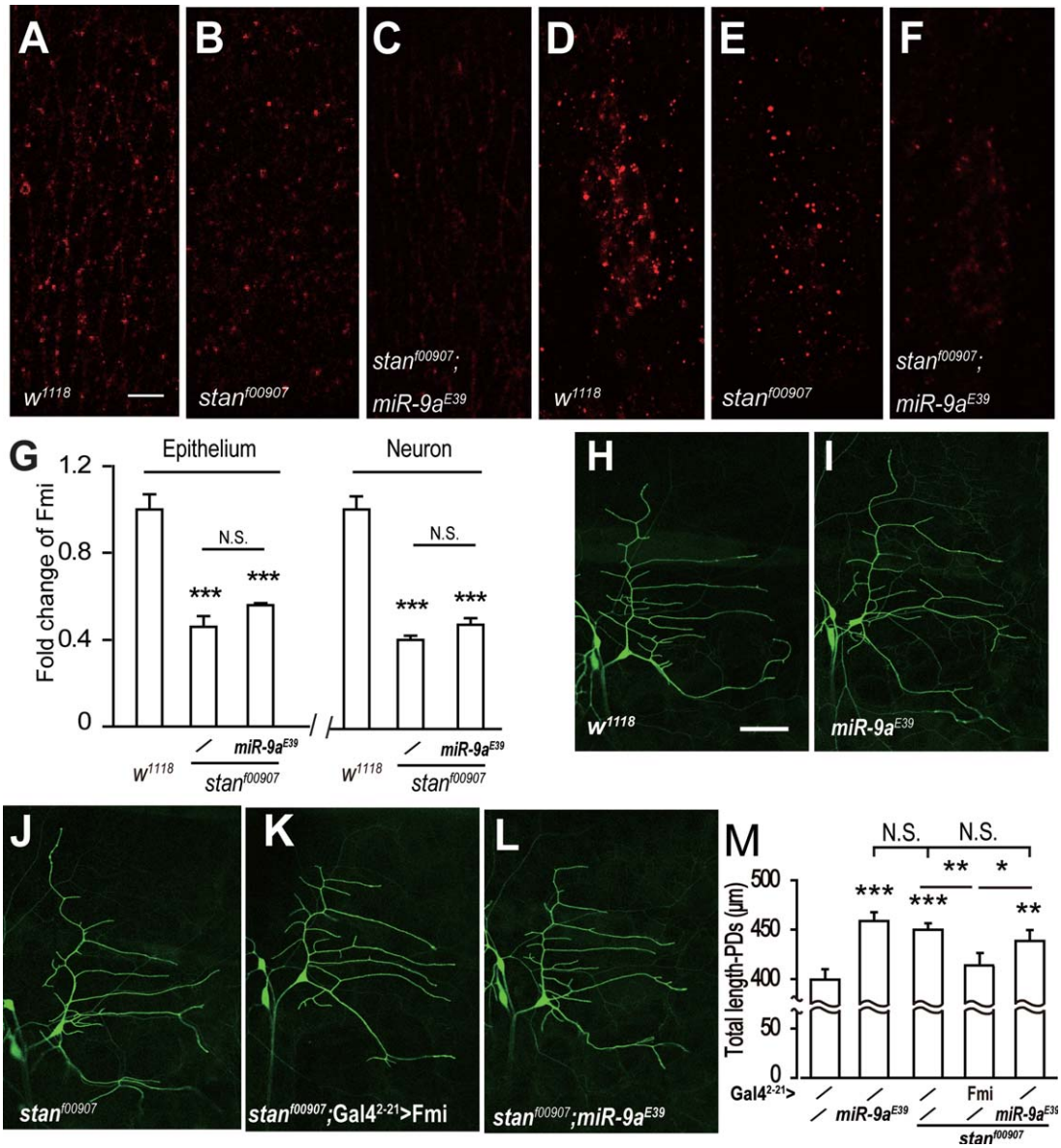

Figure 3 Neuronal Fmi is required for dendrite growth of the ddaE neuron. A–G: *fmi* mutant $stan^{f00907}$ and double mutant $stan^{f00907}$;$miR\text{-}9a^{E39}$ exhibit reduced Fmi expression in epithelia and neurons at embryonic stage 14. H–M: $stan^{f00907}$ mutants display increment in total length-PDs of ddaE neuron, which is rescued to wild type levels by overexpression of Fmi in neurons. L–M: $stan^{f00907}$;$miR\text{-}9a^{E39}$ mutants display increased total length-PDs of the ddaE neuron. Scale bar, 5 μm in A, 50 μm in F. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

resulted in a visible reduction in both total length of PDs [Fig. 2(I,L)] and length-DV [Supporting Information Fig. S3(A)]. In contrast, expressing FmiΔN (lacking the extracellular motifs) or FmiΔC (lacking the intracellular C-tail) in the neurons had no effect on dendrite growth [Supporting Information Fig. S4(A–E)], suggesting that full-length sequence of Fmi are required for its normal function in dendrite development. The causal relationship between Fmi expression level and dendrite length of ddaE neuron is summarized in Table 1.

Gq Is Involved in Fmi-Mediated Dendrite Growth

Fmi has been proposed to function as a GPCR, and our result that Fmi lacking its C-tail had no effect on

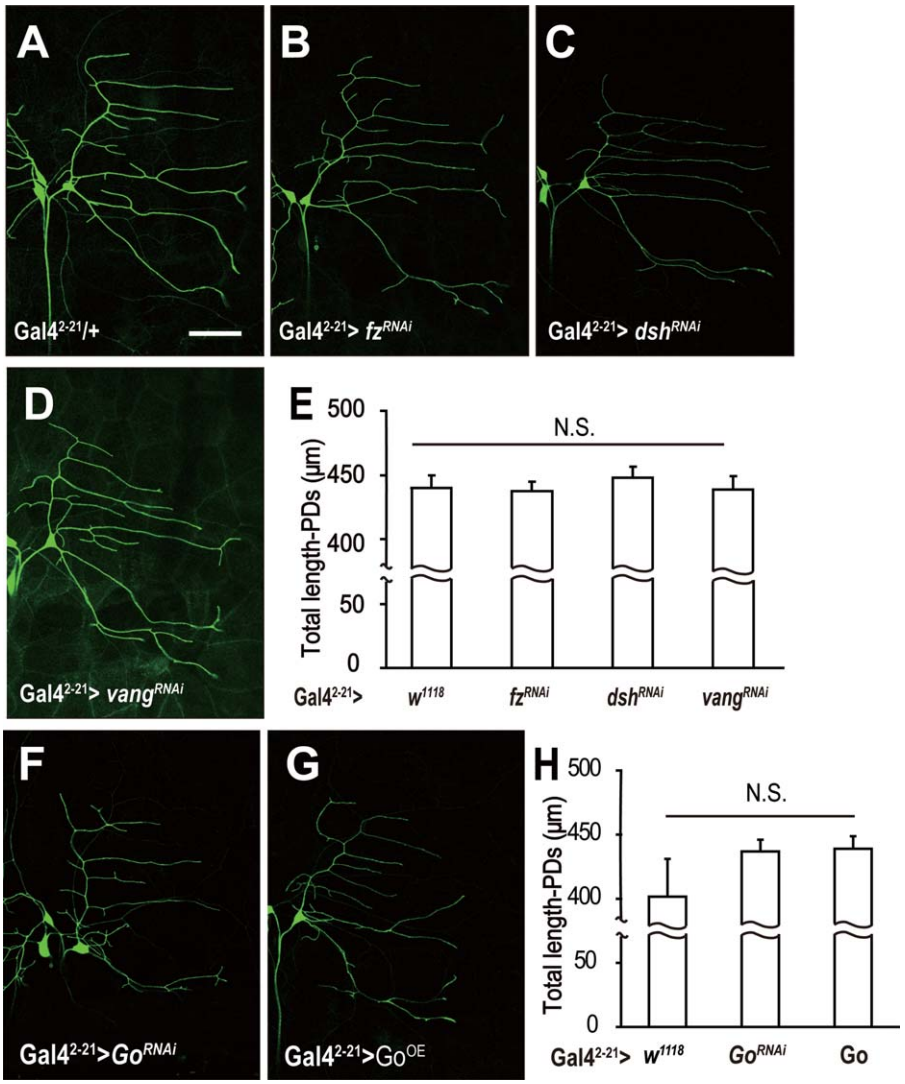

Figure 4 PCP pathway and Go are dispensable for Fmi-dependent dendrite growth. A–E: Knockdown of *fz*, *dsh*, and *vang* in neurons has no effect on the total length-PDs of the ddaE neuron. F–H: Neither overexpressing nor knocking-down Go affects the total length-PDs of ddaE neuron. Scale bar, 50 μm in A. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

dendrite growth also supported this hypothesis. The *Drosophila* heterotrimeric G protein Gαq (Gq), encoded by *Gα49B* gene, plays predominant roles in phototransduction of photoreceptors via activation of PLC and extracellular Ca^{2+} influx (Hardie et al., 2002). In addition, Gq has been found to modulate growth cone guidance (Ratnaparkhi et al., 2002). To investigate the effect of Gq on dendrite growth, we manipulated Gq levels in class I dorsal sensory neurons by overexpressing or knockingdown Gq. We found that upregulation of Gq inhibited dendrite growth, shown by a significant reduction in total length-PDs [Fig. 5(C,F)] and length-DV of ddaE neuron [Supporting Information Fig. S3(E)]. When constitutively activating Gq by overexpressing a constitutively activated form Gq^{AC} in neurons, the dendrite length of ddaE neurons significantly decreased, almost losing their general morphology [Supporting Information Fig. S5(A–D)]. In contrast, neuronal knockdown of *Gq* by Gq^{RNAi} promoted dendrite growth of ddaE neuron [Fig. 5(B,F) and Supporting Information Fig. S3(E)], suggesting that Gq, similar to Fmi, negatively regulates the dendrite growth of ddaE neurons.

The similar functions of Fmi and Gq in dendrite development led us to investigate whether Gq was

Table 1 The Causal Relationship Between Fmi-Gq Signal and Dendrite Length of the ddaE Neuron

	Fmi Expression			
Genotypes	Epithelium	Neuron	Neuronal Fmi-Gq Signal	Total Length of Primary Dendrites
miR-9a^{E39}	↑	-	(↓)*	↑
Gal4$^{2\text{-}21}$>*miR-9a*	-	↓	↓	↑
miR-9a^{E39}; Gal4^{e22c}>*miR-9a*	-	-	-	-
miR-9a^{E39}; Gal4^{e22c}> *fmi*^{RNAi}	-	-	-	-
Gal4^{e22c}>Fmi	↑	-	(↓)*	↑
Gal4$^{2\text{-}21}$>*fmi*^{RNAi}	-	↓	↓	↑
Gal4$^{2\text{-}21}$>Fmi	-	↑	↑	↓
Gal4$^{2\text{-}21}$>Fmi△N	-	△N↑	-	-
Gal4$^{2\text{-}21}$>Fmi△C	-	△C↑	-	-
stan^{f00907}	↓	↓	↓	↑
stan^{f00907}; *miR-9a*^{E39}	↓	↓	↓	↑
stan^{f00907}; Gal4$^{2\text{-}21}$>Fmi	↓	-	-	-
Gal4$^{2\text{-}21}$>*Gq*^{RNAi}	-	-	↓	↑
Gal4$^{2\text{-}21}$>Gq	-	-	↑	↓
stan^{f00907}; Gal4$^{2\text{-}21}$>*Gq*^{RNAi}	↓	↓	↓	↑
stan^{f00907}; Gal4$^{2\text{-}21}$>Gq	↓	↓	-	-
miR-9a^{E39}, Gal4$^{2\text{-}21}$>*Gq*^{RNAi}	↑	-	↓	↑
miR-9a^{E39}, Gal4$^{2\text{-}21}$>Gq	↑	-	-	-

"*," represents speculation, without direct experimental evidence.
"-," represents no change.

the downstream target of Fmi, either by Gq knockdown or overexpression Gq in a *stan*^{f00907} mutant background. Genetic interaction results revealed that neuronal knockdown of *Gq* in a *stan*^{f00907} background resulted in no additional changes in total length-PDs [Fig. 5(D,F)] or length-DV of ddaE neuron [Supporting Information Fig. S3(E)], supporting the idea that Fmi and Gq were part of the same signaling pathway involved in dendrite development. Furthermore, the increases in both total length-PDs and length-DV in the *stan*^{f00907} mutant were recovered to the level of wild type by overexpressing Gq in the neuron [Fig. 5(E,F) and Supporting Information Fig.S3(E)], suggesting that Gq is the functional downstream molecule of Fmi in dendrite development.

To further analyze whether Gq was a regulatory factor downstream of *miR-9a* during dendrite growth, we performed the same genetic interaction experiment in a *miR-9a* mutant background. Our results showed that mutating *miR-9a* and neuronal knockdown of *Gq* resulted in similar dendrite overgrowth effects. Also, knockdown of *Gq* in the neurons of a *miR-9a*^{E39} mutant background did not enhance this effect [Fig. 5(G–I) and Supporting Information Fig. S3(F)]. In contrast, neuronal overexpression of Gq in a *miR-9a*^{E39} mutant background significantly suppressed dendrites overgrowth in *miR-9a*^{E39} mutant [Fig. 5(G–I) and Supporting Information Fig. S3(F)]. Together, our results suggested that Gq might mediate the intercellular signal of Fmi in neurons to suppress dendrite growth. The causal relationship between the intensity of Fmi-Gq signal and dendrite length of ddaE neuron is summarized in Table 1.

DISCUSSION

In this study, we show that *miR-9a* down-regulates Fmi in epithelial cells, thereby regulating dendrite growth in a non-cell autonomous manner. We further show that this intercellular regulation is mediated by the inhibitory function of neuronal Fmi on dendrite growth and potential competing binding function of epithelial Fmi. Moreover, our results suggest that neuronal G protein Gq, but not Go, may function downstream of Fmi to negatively regulate dendrite growth.

miR-9 is a conserved and nervous system-enriched miRNA, with diverse and context-dependent functions during development (Yuva-Aydemir et al., 2011). Specifically, during *Drosophila* neurogenesis, *miR-9a* down-regulates Sens in epithelia, thereby suppressing the neuronal fate and regulating the specification of NPCs (Li et al., 2006). Here, we report that *miR-9a* is also absent in postmitotic sensory neurons in *Drosophila* embryos but regulates dendrite

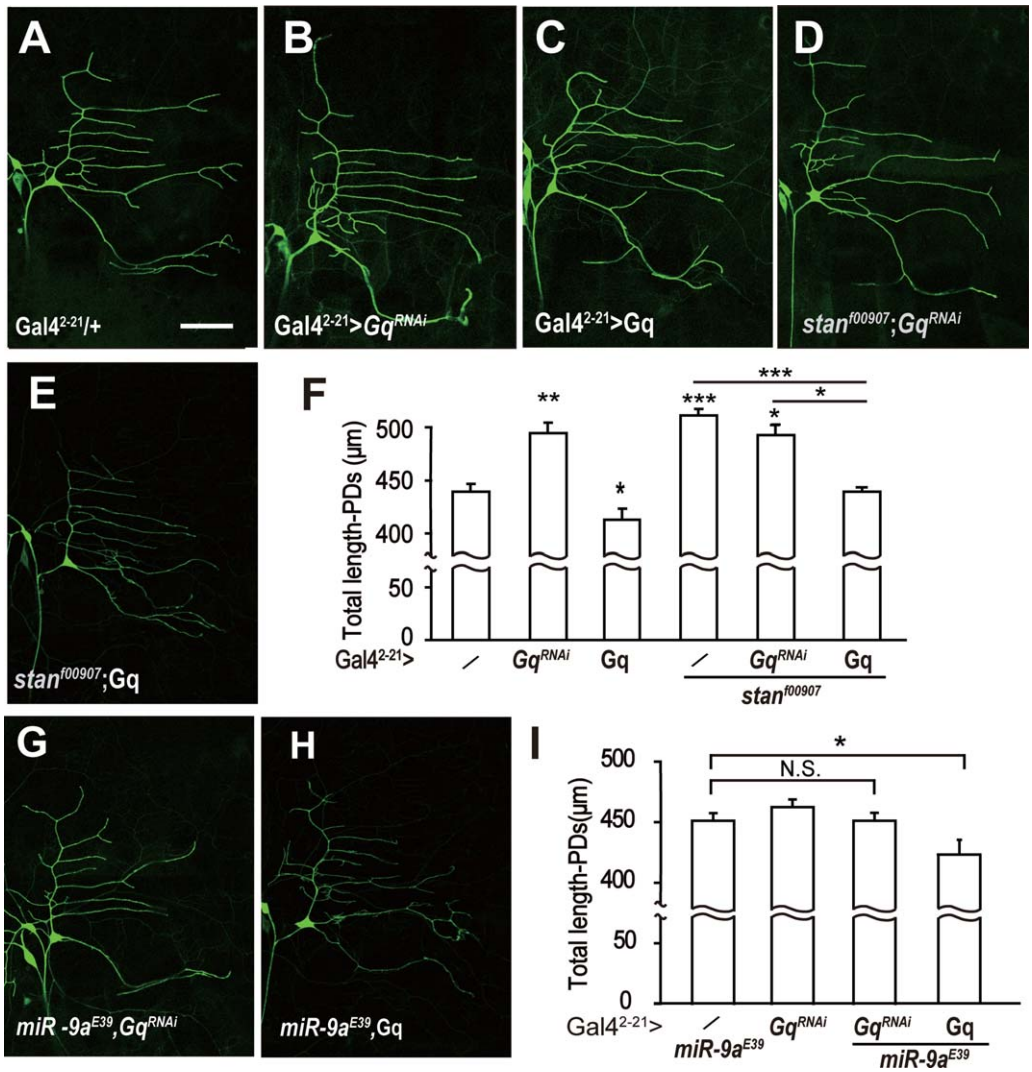

Figure 5 Gq is involved in Fmi-mediated dendrite growth. A–B and F: Knockdown of *Gq* in the neurons significantly increases the total length-PDs of the ddaE neuron. C and F: Upregulation of Gq in neurons inhibits total length-PDs of the ddaE neuron. D and F: Knockdown of *Gq* in a $stan^{f00907}$ background has no further effects on total length-PDs of the ddaE neuron. E and F: Overexpression of Gq in a $stan^{f00907}$ background reduces the total length-PDs of the ddaE neuron to wild type levels. G and I: Knocking-down *Gq* in a $miR\text{-}9a^{E39}$ background has no effect on the total length-PDs of the ddaE neuron. H-I: Upregulation of Gq in a $miR\text{-}9a^{E39}$ background reduces the total length-PDs of the ddaE neuron. Scale bar, 50 μm in A. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

growth in a non-cell autonomous manner. A similar phenomenon was reported for the *Drosophila* miRNA *ban*, which is also expressed in epithelia cells and non-cell autonomously controls the dendrite scaling of class IV sensory neurons (Parrish et al., 2009). However, the direct target of *ban* in this regulation has not been identified. Here, we reveal that *miR-9a* represses the epithelial expression of Fmi, which regulates dendrite development through cross-talking to neuronal Fmi (Fig. 6).

Cadherin repeats in Fmi N-terminal have been reported to play a role in dendrite morphogenesis through homophilic binding (Kimura et al., 2006). However, its precise regulatory mechanisms remained unclear. In this study, we showed that overexpression of Fmi in epithelial cells and neurons results in opposite phenotypes in dendrite growth. Based on our observations, we propose that neuronal Fmi plays a suppressive role in dendrite growth, whereas epithelial Fmi has a positive effect by

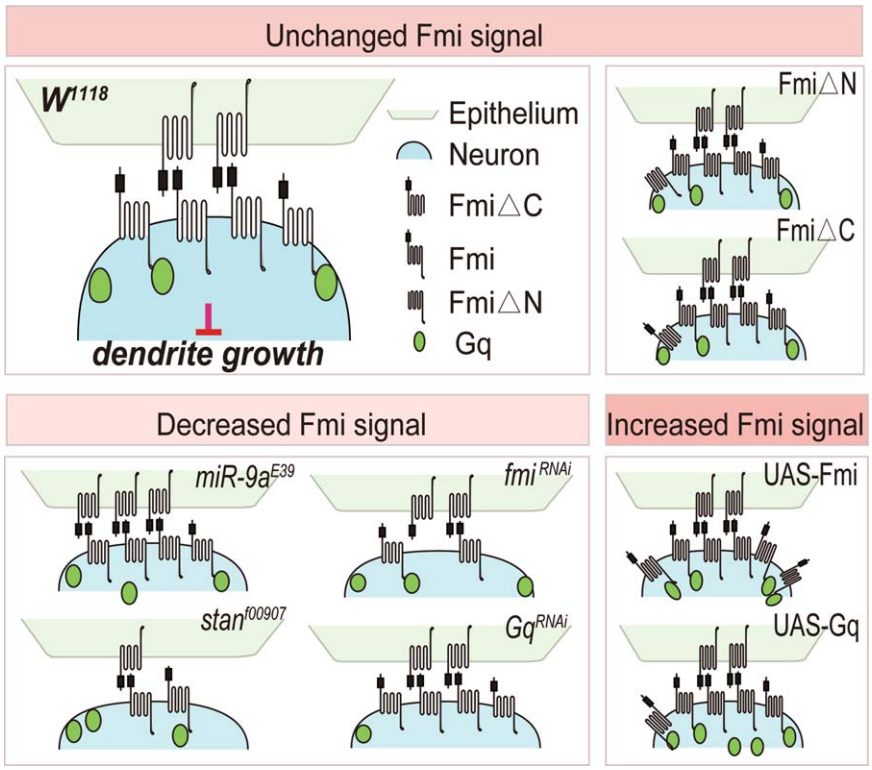

Figure 6 Model of Fmi signaling for different genetic background. In flies of Gal4$^{2\text{-}21}$>FmiΔN and Gal4$^{2\text{-}21}$>FmiΔC background, neuronal Fmi signals remain unchanged compared to wild type w^{1118}, leaving the lengths of dendrites in ddaE neurons unaffected. In *miR-9a*^{E39}, *stan*^{f00907}, Gal4$^{2\text{-}21}$>*fmi*^{RNAi}, and Gal4$^{2\text{-}21}$>*Gq*^{RNAi} background, Fmi signals in neurons are decreased and the lengths of dendrites of ddaE neuron are increased. In Gal4$^{2\text{-}21}$>UAS- Fmi and Gal4$^{2\text{-}21}$>UAS-Gq background, Fmi signals in neurons are upregulated and the lengths of dendrites of ddaE neuron are reduced. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

competing with neuronal Fmi through homophilic cadherin binding (Fig. 6). As a seven-pass transmembrane protein, Fmi shares high similarity with G-protein-coupled receptors and is thought to transduct intracellular signal and function as a receptor (Shima et al., 2007; Berger-Muller and Suzuki, 2011). Our finding that neuronal expression of Fmi lacking either the N- or the C-terminal did not affect dendrite growth, supports the hypothesis that both extracellular activation and intracellular signal transduction of Fmi are required for suppression of dendrite growth. Fmi is a core component of the PCP pathway, and has been suggested to be a co-receptor of Fz (Usui et al., 1999). Nevertheless, knockdown of other members in this pathway, such as *fz*, *vang*, and *dsh*, did not affect dendrite growth. Similar phenomena have been found in the investigation of axon path-finding in photoreceptors (Gao et al., 2000). It has been reported that G-protein Go is activated by Fz (Malbon, 2004; Wang and Malbon, 2004). However, we found that, like Fz, Go is not required for dendrite growth either. These findings suggest that Fmi regulates dendrite growth independently of the PCP pathway.

In *Drosophila*, a splice variant of *Gq*, *dgq*α*3*, functions as a component of the Robo and Frazzled signaling pathways and regulates axonal path-finding (Ratnaparkhi et al., 2002). We found that similar to neuronal Fmi, heterotrimeric G proteins Gq suppressed dendrite growth. Further genetic interactions indicated a pivotal role of Gq in mediating Fmi-dependent intracellular signaling pathways, suggesting that Gq is the G-protein that is coupled to Fmi, thus mediating the repressive function on dendrite growth.

Dendrites of ddaE neurons emerge and elongate along the D-V axis earlier than those in A-P direction (Sugimura et al., 2003). In addition, we found that Fmi expression decreased in the neuron at the later embryonic stages (data not shown). Therefore, *miR-9a* function in dendrite growth might be of greater importance in early developmental stages. This might be the reason that the overgrowth phenotype in the *miR-9a* mutant is only

found in the D-V, not, however, in the A-P direction. During *Drosophila* development, dendrites of sensory neurons exhibit a proportional enlargement of the dendrite arbor to catch up with body growth as a whole (Parrish et al., 2009). Our results suggest that the homophilic binding between epithelial and neuronal Fmi allows the crosstalk between these two cell types. We thus proposed that the *miR-9a*\Fmi\Gaq axis may serve as a novel regulatory pathway to control dendrite growth of ddaE neurons to grow along with epithelia cells and achieve proper occupation.

In conclusion, we report a novel function of *miR-9a*, which non-cell autonomously regulates dendrite growth in postmitotic neurons by negatively regulating epithelial Fmi and thereby crosstalking to neuronal Fmi. Our results imply that Gq is a candidate G protein coupled to Fmi, thereby mediating the suppressive role of Fmi signaling in dendrite growth. Together, our findings provide novel insights into the complex regulatory mechanisms of microRNAs in dendrite development, and further reveal the interplay between the different roles of Fmi functioning as cadherin and GPCR.

The authors are grateful to all the participants and those kindly provide fly strains in this study.

REFERENCES

Ambros V. 2001. microRNAs: Tiny regulators with great potential. Cell 107:823–826.

Bartel DP. 2009. MicroRNAs: Target recognition and regulatory functions. Cell 136:215–233.

Bejarano F, Smibert P, Lai EC. 2010. miR-9a prevents apoptosis during wing development by repressing Drosophila LIM-only. Dev Biol 338:63–73.

Berger-Muller S, Suzuki T. 2011. Seven-pass transmembrane cadherins: Roles and emerging mechanisms in axonal and dendritic patterning. Mol Neurobiol 44:313–320.

Bonev B, Pisco A, Papalopulu N. 2011. MicroRNA-9 reveals regional diversity of neural progenitors along the anterior-posterior axis. Dev Cell 20:19–32.

Coolen M, Thieffry D, Drivenes O, Becker TS, Bally-Cuif L. 2012. miR-9 controls the timing of neurogenesis through the direct inhibition of antagonistic factors. Dev Cell 22:1052–1064.

Dajas-Bailador F, Bonev B, Garcez P, Stanley P, Guillemot F, Papalopulu N. 2012. microRNA-9 regulates axon extension and branching by targeting Map1b in mouse cortical neurons. Nat Neurosci

Gao FB, Kohwi M, Brenman JE, Jan LY, Jan YN. 2000. Control of dendritic field formation in Drosophila: The roles of flamingo and competition between homologous neurons. Neuron 28:91–101.

Hardie RC, Martin F, Cochrane GW, Juusola M, Georgiev P, Raghu P. 2002. Molecular basis of amplification in Drosophila phototransduction: Roles for G protein, phospholipase C, and diacylglycerol kinase. Neuron 36: 689–701.

Iyengar BR, Choudhary A, Sarangdhar MA, Venkatesh KV, Gadgil CJ, Pillai B. 2014. Non-coding RNA interact to regulate neuronal development and function. Front Cell Neurosci 8:47

Jan YN, Jan LY. 2010. Branching out: mechanisms of dendritic arborization. Nat Rev Neurosci 11:316–328.

Jian Q, An Q, Zhu D, Hui K, Liu Y, Chi S, Li C. 2014. MicroRNA 340 is involved in UVB-induced dendrite formation through the regulation of RhoA expression in melanocytes. Mol Cell Biol 34:3407–3420.

Kimura H, Usui T, Tsubouchi A, Uemura T. 2006. Potential dual molecular interaction of the Drosophila 7-pass transmembrane cadherin Flamingo in dendritic morphogenesis. J Cell Sci 119:1118–1129.

Lee K, Kim JH, Kwon OB, An K, Ryu J, Cho K, Suh YH, Kim HS. 2012. An activity-regulated microRNA, miR-188, controls dendritic plasticity and synaptic transmission by downregulating neuropilin-2. J Neurosci 32: 5678–5687.

Li Y, Wang F, Lee JA, Gao FB. 2006. MicroRNA-9a ensures the precise specification of sensory organ precursors in Drosophila. Genes Dev 20:2793–2805.

Magill ST, Cambronne XA, Luikart BW, Lioy DT, Leighton BH, Westbrook GL, Mandel G, et al. 2010. microRNA-132 regulates dendritic growth and arborization of newborn neurons in the adult hippocampus. Proc Natl Acad Sci USA 107:20382–20387.

Malbon CC. 2004. Frizzleds: New members of the superfamily of G-protein-coupled receptors. Front Biosci 9: 1048–1058.

Parrish JZ, Xu P, Kim CC, Jan LY, Jan YN. 2009. The microRNA bantam functions in epithelial cells to regulate scaling growth of dendrite arbors in drosophila sensory neurons. Neuron 63:788–802.

Ratnaparkhi A, Banerjee S, Hasan G. 2002. Altered levels of Gq activity modulate axonal pathfinding in Drosophila. J Neurosci 22:4499–4508.

Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. 2006. A brain-specific microRNA regulates dendritic spine development. Nature 439: 283–289.

Shima Y, Kawaguchi SY, Kosaka K, Nakayama M, Hoshino M, Nabeshima Y, Hirano T, et al. 2007. Opposing roles in neurite growth control by two seven-pass transmembrane cadherins. Nat Neurosci 10:963–969.

Shimada Y, Usui T, Yanagawa S, Takeichi M, Uemura T. 2001. Asymmetric colocalization of Flamingo, a seven-pass transmembrane cadherin, and Dishevelled in planar cell polarization. Curr Biol 11:859–863.

Siegel G, Obernosterer G, Fiore R, Oehmen M, Bicker S, Christensen M, Khudayberdiev S, et al. 2009. A

functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis. Nat Cell Biol 11: 705–716.

Stark A, Brennecke J, Bushati N, Russell RB, Cohen SM. 2005. Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution. Cell 123:1133–1146.

Steimel A, Wong L, Najarro EH, Ackley BD, Garriga G, Hutter H. 2010. The Flamingo ortholog FMI-1 controls pioneer-dependent navigation of follower axons in C. elegans. Development 137:3663–3673.

Strutt DI. 2002. The asymmetric subcellular localisation of components of the planar polarity pathway. Semin Cell Dev Biol 13:225–231.

Sugimura K, Yamamoto M, Niwa R, Satoh D, Goto S, Taniguchi M, Hayashi S, et al. 2003. Distinct developmental modes and lesion-induced reactions of dendrites of two classes of Drosophila sensory neurons. J Neurosci 23:3752–3760.

Thibault ST, Singer MA, Miyazaki WY, Milash B, Dompe NA, Singh CM, Buchholz R, et al. 2004. A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat Genet 36:283–287.

Usui T, Shima Y, Shimada Y, Hirano S, Burgess RW, Schwarz TL, Takeichi M, et al. 1999. Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. Cell 98:585–595.

Vo NK, Cambronne XA, Goodman RH. 2010. MicroRNA pathways in neural development and plasticity. Curr Opin Neurobiol 20:457–465.

Wang HY, Malbon CC. 2004. Wnt-frizzled signaling to G-protein-coupled effectors. Cell Mol Life Sci 61:69–75.

Wayman GA, Davare M, Ando H, Fortin D, Varlamova O, Cheng HY, Marks D, et al. 2008. An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. Proc Natl Acad Sci USA 105: 9093–9098.

Wu JS, Luo L. 2006. A protocol for dissecting Drosophila melanogaster brains for live imaging or immunostaining. Nat Protoc 1:2110–2115.

Yuva-Aydemir Y, Simkin A, Gascon E, Gao FB. 2011. MicroRNA-9: Functional evolution of a conserved small regulatory RNA. RNA Biol 8:557–564.